Transcriptome Sequencing Provides Evidence of Genetic Assimilation in a Toad-Headed Lizard at High Altitude

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Abstract Understanding how organisms adapt to the environment is a compelling question in modern evolutionary biology. Genetic assimilation provides an alternative hypothesis to explain adaptation, in which phenotypic plasticity is first triggered by environmental factors, followed by selection on genotypes that reduce the plastic expression of phenotypes. To investigate the evidence of genetic assimilation in a high-altitude dweller, the toad-headed agama *Phrynocephalus vlangalii*, we conducted a translocation experiment by moving individuals from high- to low-altitude environments. We then measured their gene expression profiles by transcriptome sequencing in heart, liver and muscle, and compared them to two low-altitude species *P. axillaris* and *P. forsythii*. The results showed that the general expression profile of *P. vlangalii* was similar to its viviparous relative *P. forsythii*, however, the differentially expressed genes in the liver of *P. vlangalii* showed a distinct pattern compared to both the low-altitude species. In particular, several key genes (FASN, ACAA2 and ECI2) within fatty acid metabolic pathway were no longer differentially expressed in *P. valgnalii*, suggesting the loss of plasticity for this pathway after translocation. This study provides evidence of genetic assimilation in fatty acid metabolism that may have facilitated the adaptation to high-altitude for *P. vlangalii*.

Keywords genetic assimilation, gene expression, plasticity, *Phrynocephalus vlangalii*

1. Introduction

Elucidating the mechanisms of adaptation is a compelling question in modern evolutionary biology (Rose, 2001; Smith and Eyre-Walker, 2002). In the past decades, a "mutation-first evolution" model was widely accepted to explain the process of adaptation, in which mutations linked to fit phenotypes are under natural selection, resulting in changes in phenotype frequencies, and ultimately, adaptation (Carroll, 2008). However, a "plasticity-first evolution" model has also been proposed in which phenotypic plasticity is first triggered by environmental factors, followed by selection on genotypes influencing the plastic expression of phenotypes through genetic accommodation (Moczek *et al.*, 2011; Jones and Robinson, 2018). Genetic assimilation is a special case of such process, where the initially plastic phenotypes are gradually fixed, leading to reduced phenotypic plasticity and only adaptive phenotypes, even without environmental stimuli (Waddington, 1953; Pigliucci, 2006). Although a number of studies have shown evidence of genetic assimilation, such as cases in *Drosophila melanogaster* (Dworkin, 2005; Ghalambor *et al.*, 2010) and *Spea bombifrons* (Levis and Pfennig, 2019), the role of genetic assimilation in adaptive evolution remains unclear and controversial.

Gene expression profiles provide an excellent tool to investigate genetic assimilation in evolution. In general, phenotypic plasticity springs from environmentally sensitive regulation of gene expression, which alters the profiles of gene expression (Colinet and Hoffmann, 2012; Beaman *et al.*, 2016). As plastic phenotypes may evolve in response to selection with increased or decreased levels of plasticity, the gene expression associated with the phenotypes may evolve as well (Renn and Schumer, 2013). Thus, variable patterns of gene expression mirror the evolution of phenotypic plasticity. In the case of genetic assimilation, plastically expressed genes in response

to the environment may finally become fixed (Scoville and Pfrender, 2010; Renn and Schumer, 2013). Previous studies that have used gene expression profiles as a tool to investigate the pattern of genetic assimilation have mainly focused on eurociality in the honeybee (Toth *et al.*, 2007; Bloch and Grozinger, 2011); host specialization in *Drosophila* (Matzkin *et al.*, 2006; Matzkin, 2012); and character displacement in spadefoot toads (Levis *et al.*, 2017).

Toad-headed agamas (genus *Phrynocephalus*) at the Qinghai-Tibetan Plateau (Huey, 1982; Zhao *et al.*, 1982) provide an

excellent model system to study genetic assimilation. As true dwellers of high-altitude environments, as high as 5 300 m above sea level (a.s.l), this group has experienced a long-term adaptive evolution under several extreme stressors, including hypobaric hypoxia, low ambient temperatures, and strong UV radiation (Scheinfeldt and Tishkoff, 2010; Cheviron and Brumfield, 2011). Phylogenetic studies indicated that a total of six high-altitude species in this genus formed a monophyletic viviparous clade, including another low-altitude species, *P. forsythii* (Figure 1A; Guo *et al.*, 2002; Guo and Wang, 2007;
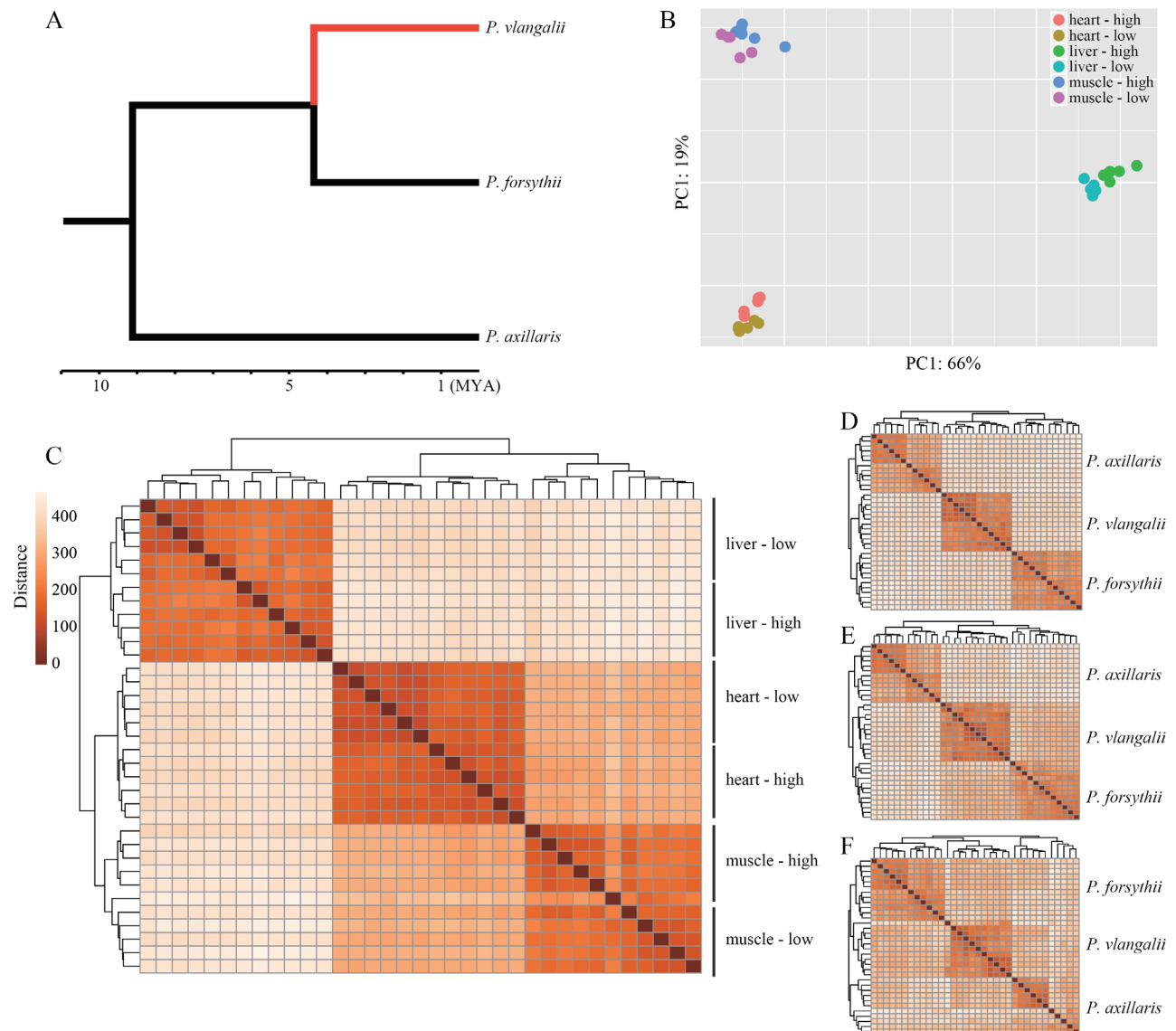

Figure 1 Translocation experiment and transcriptome sequencing. A: The phylogenetic relationship among *Phrynocephalus axillaris*, *P. forsythii*, and *P. vlangalii*. The red branch indicates the viviparous high-altitude clade leading to *P. vlangalii*. B: Principal component analysis (PCA) plot for gene expression profiles. C: Heatmap for gene expression profiles among *P. vlangalii* samples. D, E, and F: Heatmap for gene expression profiles among the three species for heart, liver, and muscle, respectively. In all the comparisons, *P. vlangalii* was clustered with *P. forsythii*, consistent with their phylogenetic relationship.

Wang *et al.*, 2014).

High-altitude toad-headed agamas have evolved a series of characteristics that underline their adaptations to extremely high-altitude environments. At DNA sequence level, a couple of genes have been identified with signature of positive selection associated with energy metabolism and DNA repair (Yang *et al.*, 2014; Yang *et al.*, 2015; Sun *et al.*, 2018). Accordingly, physiological studies revealed that, compared to low-altitude species, high-altitude toad-headed agamas may have decreased basal metabolic rate and increased the utilization of nutrients (e.g. fatty acid) to balance the energy budget (Tang *et al.*, 2013; Li *et al.*, 2016; Zhang *et al.*, 2018), which is a common strategy for high-altitude adaptation for ectothermic vertebrates (Cooper *et al.*, 2002; Li *et al.*, 2016). On the other hand, low-altitude toad-headed agamas exhibited the same direction of plastic response to highland environments (e.g. Tang *et al.*, 2013). Qi *et al.* (unpublished) have conducted an experiment by translocating two low-altitude species *P. axillaris* (oviparous) and *P. forsythii* (viviparous) to highland environments and measured their transcriptomic, metabolomic, and behavioral responses. The two species showed a similar pattern, in which significantly plastic change occurred for core genes and metabolites within fatty acid metabolic pathway. However, whether the same plasticity still exists in high-altitude agamas remains unknown. For other taxa, several species have shown loss of plasticity while adapting to high-altitudes due to genetic assimilation, such as *Alnum glutinosa* (Kort *et al.*, 2016), and montane butterfly *Colias eriphyle* (Kingsolver and Buckley, 2017). Therefore, to investigate if toad-headed lizards have experienced the loss of plasticity at high-altitudes could provide new evidence of genetic assimilation in high-altitude adaptation for ectotherms.

Here, we present a study to investigate the gene expression profiles and plasticity of a high-altitude species Qinghai toad-headed agama (*P. vlangalii*). This species inhabits in Qinghai-Tibetan Plateau at altitudes ranging from 2000 to 4600 a.s.l. (Zhao *et al.*, 1999). We implemented a translocation experiment by moving *P. vlangalii* individuals from high- to low-altitude environment and measured the gene expression profiles for three organ tissues - heart, liver, and muscle, to investigate the plastic changes in gene expression. In addition, we compared *P. vlangalii* to two low-altitude species, *P. axillaris* (oviparous) and *P. forsythii* (viviparous), and revealed the pattern of expression plasticity for *P. vlangalii* that may indicate whether genetic assimilation has contributed to theadaptation to high-altitude environmentfor this species.

2. Materials and Methods

2.1. Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals

were strictly followed. All animal sample collection protocols complied with the current laws of China. The sampling and experiment in this study were carried out with permission (Number 2017005) from the Ethical Committee for Animal Experiments in Chengdu Institute of Biology, Chinese Academy of Sciences.

2.2. Translocation experiment A total of 12 male adults of *P. vlangalii* were sampled from Zoige, Sichuan Province of China, at an altitude of 3 400 meters a.s.l. All samples were randomly and evenly assigned into an 'origin group' and 'translocation group'. For the origin group, we measured their morphological traits and then performed euthanasia using decapitation and collected tissues of heart, liver, and muscle, by preserved in RNA later (Invitrogen, USA) in the field. For the translocation group, we moved the individuals to a workstation in Chengdu City, Sichuan Province of China, with an altitude of 650 meters a.s.l. All individuals had been kept in an outdoor enclosure similar to their native environment for six weeks to make the individuals acclimate to translocation environment. Then, the same procedure was conducted for the translocation group to collect tissue samples. To alleviate the potential bias stimulated by the environment, the field sites were standardized in three aspects: 1) sand obtained from the origin sites of *P. vlangalii* to the translocation site as substrate; 2) mealworms were provided as food every three days for both sites; and 3) fishing net above enclosures to reduce the risks of bird predation.

2.3. Transcriptome sequencing Total RNA was extracted from each tissue sample according to TRIzol protocols (Invitrogen, USA). Transcriptome sequencing was implemented on Illumina HiSeq 2500 platform with paired-end 150 base pair (bp) by Novogene (Beijing, China). Sequence data were deposited in NCBI Short Reads Archive (SRA) with BioProject accession number PRJNA718616. Raw sequence reads were first cleaned by removing the adapter sequences and low-quality base calls using a Novogene pipeline. Trimmomatic v0.35 (Bolger *et al.*, 2014) was used to trim the reads with LEADING:3, TRAILING:3, SLIDINGWINDOW:4:5, MINLEN:70, and default parameters. We checked for reads quality before and after filtering with FASTQC version 0.11.8 (Andrews, 2010). One individual with all three types of tissues (E1, K1, and Q1; details in Table S1) was used for *de novo* assembly of transcriptome via Trinity v2.8.4 after *in silico* read normalization (Grabherr *et al.*, 2011; Haas *et al.*, 2013). We then used kallisto version 0.44.0 (Bray *et al.*, 2016) to quantify the abundance of the assembly and build the transcripts expression matrices. Assembled transcripts with 'transcripts per million transcripts' (TPM) less than three were filtered to generate the final assembly for each species. To compare to other two low-altitude species *P. axillaris* and *P. forsythii* (Qi *et al.*, unpublished), a best reciprocal hit (BRH)

method was applied to identify 1:1:1 orthologous sequences among the three species (Camacho *et al.*, 2009).

2.4. Differential expression analysis The clean reads for each sample were mapped against the *P. vlangalii* transcriptome assembly by using STAR version 2.6 (Dobin *et al.*, 2013). The number of reads matched to the same transcripts was counted by HTSeq-count tool with the 'union' resolution mode (Anders *et al.*, 2015). The overall similarity among tissue samples was measured by Euclidean distance and visualized by clustering heatmap through 'pheatmap' package after regularized log transformation (rlog) of normalized counts via DESeq2 version 1.20 (Love *et al.*, 2014). Principal component analysis (PCA) was also used to assess the relationship among different samples. Differentially expressed genes (DEGs) were estimated through generalized linear models in edgeR package version 3.22.5 (Robinson *et al.*, 2010; McCarthy *et al.*, 2012). We adopted a strict criterion to identify DEGs, with fold-value ≥ 2 and adjusted *P*-value (FDR) < 0.05.

Functional annotation was performed by mapping the transcripts against the UniProtKB/Swiss-Prot database (release "2018_08") with blast hits E-value cut-off greater than 10^{-5}. For those transcripts with annotation information, functional over-representations of DEGs were performed using the clusterProfiler package (Yu *et al.*, 2012) in R with annotation to GO category and KEGG pathway database. The minimum number of genes required for each test of a given category was 5. All tests were corrected by false discovery rate (FDR). To compare the plastic pattern with *P. axillaris* and *P. forsythii* for genes associated with fatty acid metabolism, we also checked the expression profiles of 12 diagnostic genes (Table S2), which showed differential expression in liver for the two low-altitude species, including key genes regulating the synthesis (Fatty Acid Synthase, FASN) and catabolic process (Acetyl-Coenzyme A Acyltransferase 2, ACAA2, and Enoyl-CoA Delta Isomerase 2, ECI2) of fatty acid. We calculated the absolute fold change of expression from low- to high-altitude for those genes among the three species, and tested the significance of differences by two-tailed Student's *t* test.

3. Results

3.1. Transcriptome sequencing A total of 44 793 768–70 718 484 raw reads were generated for *P. vlangalii* by Illumina sequencing. After filtering, 42 722 956–67 578 714 reads were retained. One sample (R6) was excluded from the following analyses due to low quality of sequencing. Overall, 36 336 transcripts were obtained with N50 size of 2 232 bp and mean length of 1 093 bp. By BRH method, 8 892 orthologous transcripts were identified among *P. axillaris*, *P. forsythii*, and *P. vlangalii*.

3.2. Gene expression profile Both principal component analysis (PCA) and clustering analysis demonstrated that each tissue type presented a distinct expression signature and all samples were unambiguously grouped by tissue origin (Figure 1B). Within each tissue, samples were also separated by origin and translocation groups. In addition, samples from heart and muscle expressed closer than from liver, which may reflect that a large part of the heart is composed of cardiac muscle tissue (Figure 1C). By comparing the expression profiles of *P. vlangalii* to the other two low-altitude species *P. axillaris* and *P. forsythii*, we found that the *P. vlangalii* was closer to *P. forsythii* in all the three tissues, consistent with their phylogenetic relationship (Figure 1D, E, F).

3.3. Differential expression analysis Given low-altitude samples as reference, a total of 875 differentially expressed genes (DEGs) were identified in the heart for *P. vlangalii*, with 400 down-regulated and 475 up-regulated. Similarly, 688 DEGs were identified in muscle, with 345 down-regulated and 343 up-regulated. We identified 1 220 DEGs in liver, the greatest number of DEGs among all three tissues, with 612 down-regulated and 608 up-regulated.

Through functional annotation, we found that no GO category and KEGG pathway was over-represented by DEGs in heart. A total of 41 GO categories and 13 KEGG pathways were identified over-represented by DEGs in liver (Figure S1). Network clustering of the GO categories indicated that most of the categories were associated with monocarboxylic acid metabolic process, which could be linked to antibiotic catabolic process and detoxification (Figure 2A). In muscle, 7 GO categories and 1 KEGG were over-represented by DEGs (Figure S2), which were associated with extracellular matrix organization and carbohydrate derivative catabolic process.

For the 12 diagnostic genes associated with fatty acid metabolism, the absolute fold change of expression in liver of *P. vlangalii* was significantly lower ($\Delta \log_2 FC = 0.8300$) than that of *P. axillaris* ($\Delta \log_2 FC = 2.1298$; *P-value* = 2.84×10^{-6}) and *P. forsythii* ($\Delta \log_2 FC = 1.7397$; *P-value* = 1.31×10^{-6}), along with no significant difference between the latter two species (*P-value* = 0.44) (Figure 2B). In addition, none of the key genes FASN, ACAA2 and ECI2 was differentially expressed between low- and high- altitude (Figure 2C).

4. Discussion

In this study, we conducted a translocation experiment by moving *P. vlangalii* from a high- to low-altitude environment and measured their expression profiles and plasticity via transcriptome sequencing. Our results clearly illustrated that each tissue type of *P. vlangalii* presented an unambiguous expression signature, with hundreds of DEGs up-regulated or
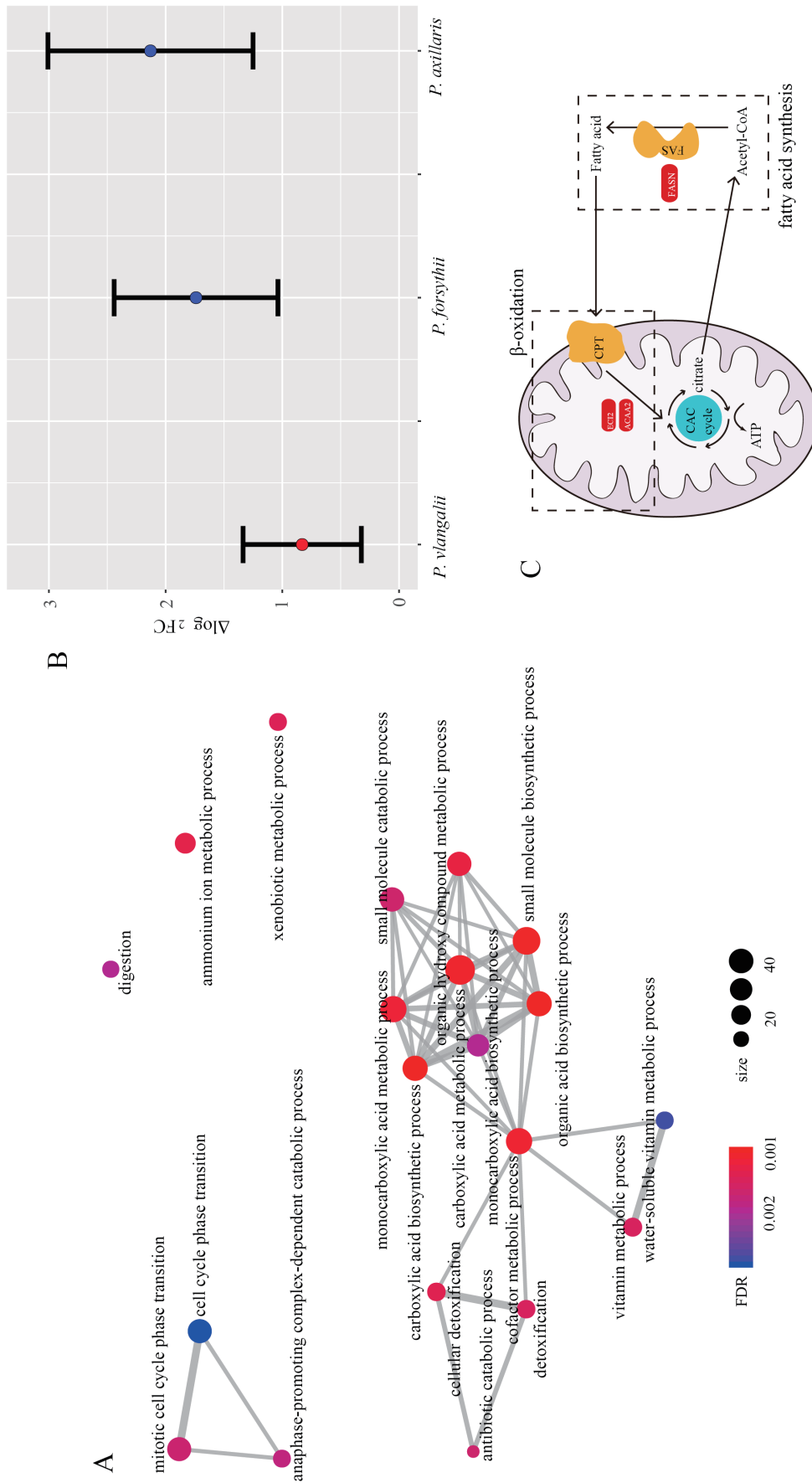
Figure 2 Functional analysis for differentially expressed genes in liver. (A) Network clustering of the GO categories over-represented by DEGs. The circles indicate the GO categories, where the grey lines represent the connection between two categories by shared DEGs. The clusterings revealed that the categories linked to antibiotic catabolic process and detoxification were over-represented by DEGs. (B) The mean fold-change values of 12 diagnostic genes associated with fatty acid metabolism between low- and high-altitude environments for the three toad-headed lizard species. (C) The schematic diagram of fatty acid metabolism. The red squares represent the functions that the key genes involve in.

down-regulated in each tissue, respectively. In comparison to the other two low-altitude species *P. axillaris* (oviparous) and *P. forsythii* (viviparous), although the expression profile of *P.vlangalii* was still closer to *P. forsythii*, DEGs in liver exhibited a distinct pattern from the other two species, with reduced plasticity in expression of genes associated with fatty acid metabolism.

A common strategy for ectothermic vertebrates in response to extreme environments at high-altitude is to suppress basal metabolism and increase utilization of nutrients to balance the energy budget (Cooper *et al.*, 2002; Li *et al.*, 2016; Zhang *et al.*, 2018). Tang *et al.* (2013) revealed that a high-altitude toad-headed agama, *P. erythrurus*, behaved similarly, with lower mitochondrial respiratory rate but higher fat utilization in liver compared to a low-altitude species *P. przewalskii*. Qi *et al.* (unpublished) further found that both oviparous (*P. axillaris*) and viviparous (*P. forsythii*) species in this genus showed a very similar plastic pattern of gene expression and metabolites associated with fatty acid metabolism in liver by translocating from low- to high-altitude. However, our study suggests that the true high-altitude species *P. vlangalii* has a distinct pattern. Although the general expression profile of *P. vlangalii* was still closer to *P. forsythii*, consistent with their phylogenetic relationship, DEGs in liver of *P. vlangalii* were mostly concentrated in functional categories like monocarboxylic acid metabolic process, antibiotic catabolic process and detoxification. Furthermore, the expression patterns of 12 diagnostic genes associated with fatty acid metabolism, which showed significantly differentially expression in *P. axillaris* and *P. forsythii*, illustrated reduced plastic expression in *P. vlangalii*, including key genes FASN, ACAA2, and ECI2. FASN gene encodes an enzyme fatty acid synthase that plays a core role in fatty acid synthesis (Jayakumar *et al.*, 1995); ACAA2 and ECI2 encode proteins which are key mitochondrial enzymes involved in beta-oxidation, a step in the catabolic process of fatty acid (Abe *et al.*, 1993; Geisbrecht *et al.*, 1999). None of these genes were differentially expressed between low- and high-altitude groups for *P. vlangalii*, suggesting no significant difference in fatty acid metabolism after translocation. The result indicated that *P. vlangalii* may have lost the capacity of plasticity in fatty acid metabolism due to genetic assimilation. Plasticity in fatty acid metabolism is thought to contribute to maintaining life activities for organisms moving to high altitude (Hammond *et al.*, 2001; Storz *et al.*, 2010; Refsnider *et al.*, 2018). As a true dweller adapted to high altitude, *P. vlangalii* may have evolved other traits to trade-off the reduction of capacity for plasticity in fatty acid metabolism, similar to the case of *P. erythrurus* (Tang *et al.*, 2013). However, the detailed mechanism for *P. vlangalii* still needs further research.

Our study provided special evidence of genetic assimilation that may have facilitated the high-altitude adaptation for *P. vlangalii*. Genetic assimilation refers to the process in which initially plastic phenotypes are gradually fixed, leading to reduced phenotypic plasticity and only adaptive phenotypes, even without environmental stimuli (Waddington, 1953; Pigliucci, 2006). At gene expression level, plastically expressed genes may finally become fixed, given that gene expression mirrors phenotypic plasticity (Scoville and Pfrender, 2010; Renn and Schumer, 2013). Genetic assimilation provides an alternative hypothesis to explain the mechanism of adaptive evolution, where phenotypic plasticity is first triggered by environmental factors, followed by selection on genotypes influencing the plastic expression of phenotypes (Moczek *et al.*, 2011; Jones and Robinson, 2018). In fact, species such as *Alnum glutinosa* (Kort *et al.*, 2016) and *Colias eriphyle* (Kingsolver and Buckley, 2017) also showed similar loss of plasticity in adaptation to high-altitude environments, which indicated that the genetic assimilation might be an effective way for organisms to adapt to extreme environments. However, more studies especially on modeling of phenotypic plasticity are still required to elucidate the role of genetic assimilation in adaptation.

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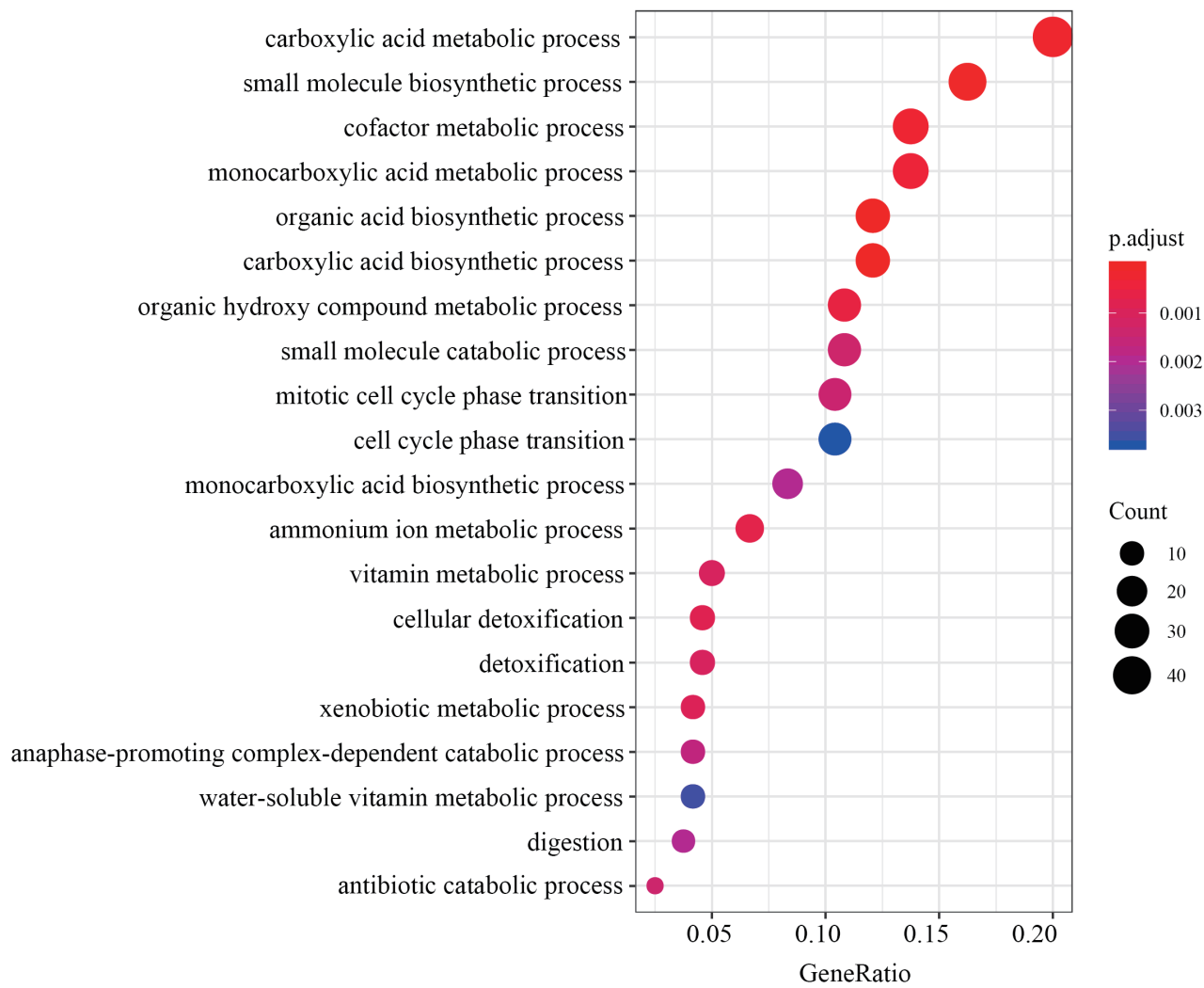**Handling Editor: Heling Zhao**

Appendix

Figure S1 Top 20 GO categories over-represented by DEGs in liver.
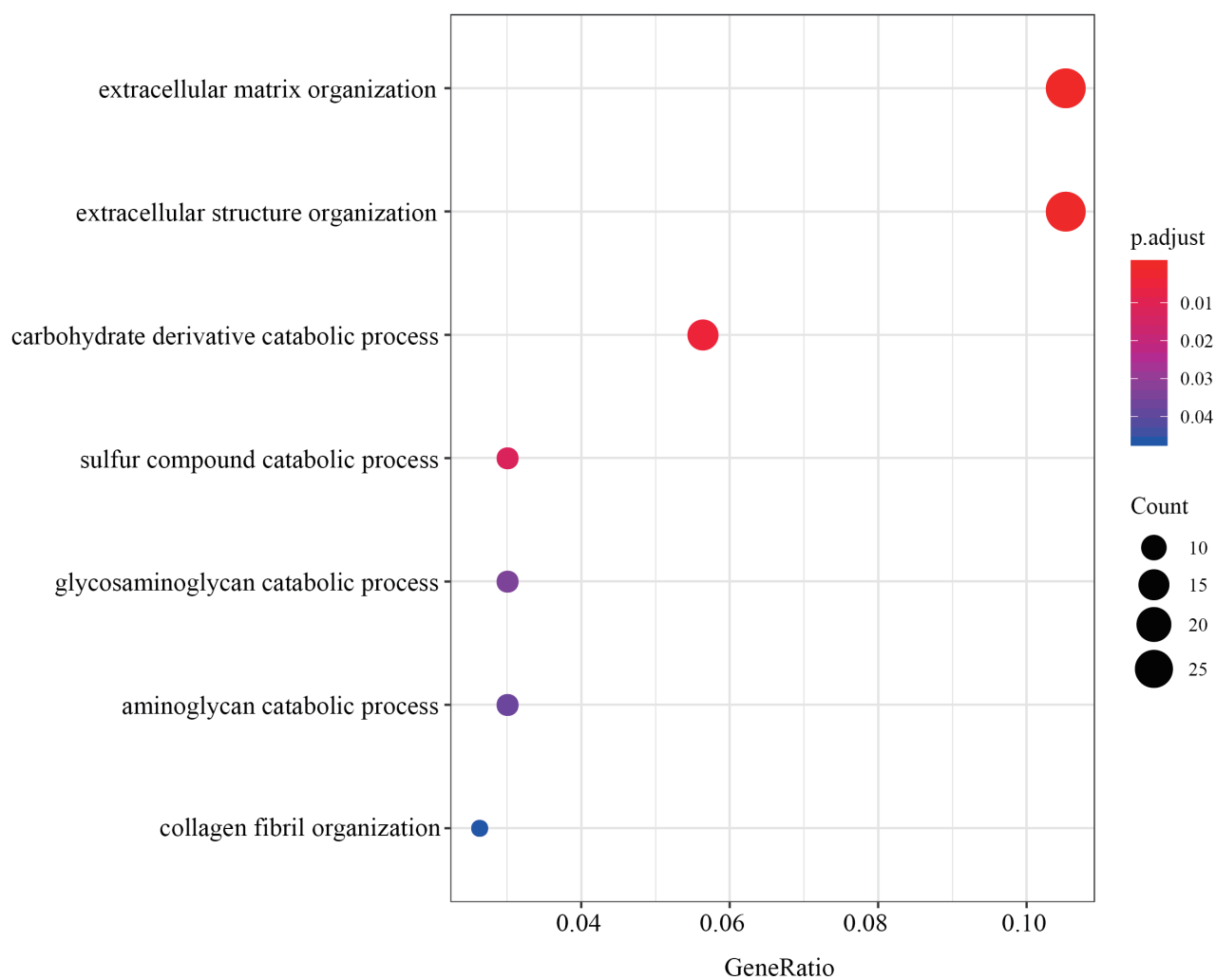
Figure S2 GO categories over-represented by DEGs in muscle.

Table S1 Sample information.

Sample ID	Tissue type	Condition
E1	Heart	High
E2	Heart	High
E3	Heart	High
E4	Heart	High
E5	Heart	High
E6	Heart	High
F1	Heart	Low
F2	Heart	Low
F3	Heart	Low
F4	Heart	Low
F5	Heart	Low
F6	Heart	Low
K1	Liver	High
K2	Liver	High
K3	Liver	High
K4	Liver	High
K5	Liver	High
K6	Liver	High
L1	Liver	Low
L2	Liver	Low
L3	Liver	Low
L4	Liver	Low
L5	Liver	Low
L6	Liver	Low
Q1	Muscle	High
Q2	Muscle	High
Q3	Muscle	High
Q4	Muscle	High
Q5	Muscle	High
Q6	Muscle	High
R1	Muscle	Low
R2	Muscle	Low
R3	Muscle	Low
R4	Muscle	Low
R5	Muscle	Low
R6	Muscle	Low

Table S2 Diagnostic genes associated with fatty acid metabolism.

Symbol	Gene name
RGN	Regucalcin
PCCB	Propionyl-CoA carboxylase beta chain
PHYH	Phytanoyl-CoA dioxygenase
ECI2	Enoyl-CoA delta isomerase 2
ACSS2	Acetyl-coenzyme A synthetase
SREBF1	Sterol regulatory element-binding protein 1
ECI1	Enoyl-CoA delta isomerase 1
CRYL1	Lambda-crystallin homolog
ACAA2	Acetyl-CoA acetyltransferase
FASN	Fatty acid synthase
CYP2U1	Cytochrome P450 2U1
ACSM3	Acyl-coenzyme A synthetase